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Fall Armyworm (*Spodoptera frugiperda*) control by RNAi as a Plausible Component to Integrated Pest Management Practices

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Abstract: Fall armyworm (*Spodoptera frugiperda*) control through RNAi machinery as a novel mode of action is a weapon sought after by the agricultural community to squelch the growing problem of *S. frugiperda* resistance to pesticides and transgenic crops. Significant gains in the understanding of RNAi cellular interactions and insect gene silencing are represented to provide opportunities to influence further research of *S. frugiperda* RNAi. This composition highlights the intracellular action of dsRNA, successful RNAi attempts on insect pests, the biological difficulty of *S. frugiperda* and how RNAi success against *S. frugiperda* would be highly advantageous in the quest to mitigate resistance regarding current integrated pest management (IPM) practices to control this polyphagous and genetically plastic agricultural pest.

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I. Prologue: Address from the Author

Insect pest control of agricultural crops has evolved significantly in the last century. I have witnessed this evolution in the company I work for. Through my education via The University of Nebraska-Lincoln I have learned an incredible amount of information. Much of it is utilized directly within my work, other components were vital in providing a tangible comprehension of the AgBiotech landscape and insect pest problems in the future. Overall, the tandem work & education relationship has had an incredible impact on my knowledge and career.

The largest contributing factor to this Degree Project was my blooming passion for Integrated Pest Management (IPM) practices when I was able to conceptualize that my entire department is focused on one small area of one crop and a small pool of insect pests that we wish to control. While I am not necessarily focused on the economics of IPM, I am interested in the plethora of options described in various texts to challenge insect pests to reduce the economic impact of their presence. The challenge and success of IPM is very real and very important. The ability to reliably integrate pest management practices, it is imperative to apply the current technology & knowledge of the pest, ecosystem, & economy researchers have at hand to make sound decisions (Pedigo & Rice, 2009). One of the newest technologies in AgBiotech is RNAi and its many uses, namely, in insect control. The successes I had discovered during previous literature reviews for classes sparked my interest in RNAi for the fall armyworm as it is a very troublesome pest that has thwarted IPM practices in a relatively short period of time.

II. Introduction

The control tactics used in integrated pest management has evolved significantly from exposing the pests (and species within a community) to copious amounts of chemical pesticides

in an effort to eradicate a pest. We quickly learned, however, this was not a very progressive tactic and led to many environmental issues. Since the days of Stern *et al* (1959) and Rachel Carson's Silent Spring (1962), we have moved towards an attempt of reducing pesticide use on our crops through other means: natural enemies of the insect pests, transgenic crops and implementation of cultural control methods. Transgenic crops have been created using various novel *Bacillus thuringiensis* genes to mitigate the damage certain pests inflict on our crops (Tabashnik *et al*, 2013). These plants express insecticidal proteins in a far more discrete manner than some chemical insecticides, which presents some benefits to the environment. This increase in target specificity possesses a logical beneficial attribute to not only growers, but to the environment when perhaps only the pest in question is controlled and not non-target organisms (NTOs).

As time has progressed from the first transgenic crop products, namely corn (*Zea mays*), growers are experiencing reduced efficacy and increased resistance in a variety of pests (Gordon & Waterhouse, 2007; Tabashnik *et al*, 2013; Huang *et al*, 2014), which for a variety of reasons, is taxing on the economic threshold and environment. One such pest, fall armyworm (*Spodoptera frugiperda*-Lepidoptera, Family: Noctuidae) has developed resistance to specific modes and sites of action of the currently marketable transgenic crops that once effectively targeted *S. frugiperda*. New modes of action (MOA), like RNA interference (RNAi), is an exciting technology that may be capable of reducing, if not eliminating, the damage *S. frugiperda* causes to economically important crops and provide some relief to NTOs due to its high target species specificity.

In the passages below, selected research literature and textbooks are utilized to formulate understanding of this new mode of action in RNAi. Additional selections describe the successes

the agricultural biotechnology community has observed in the introduction of RNAi as an insect control tactic. This information is weighted against the ever present enigma of *S. frugiperda* and the difficulties this insect species presents to propose further investigations to RNAi as a plausible component to IPM and what the current state of RNAi technology and research believes is possible considering this recalcitrant pest.

III. What is RNAi?

Mechanisms of Intracellular Interactions

Originally observed in 1990 by Napoli *et al* (1990) attempted to express an enzyme-chalcone synathase to promote vibrant color in Petunia flower petals, however, the overexpression of this enzyme effectively ceased pigment production in exposed petunias. This instance, originally described as 'co-suppression' or posttranscriptional gene silencing (PTGS), was due to the degradation of mRNA (Plasterk & Ketting, 2000; Whangbo & Hunter, 2008) which stops a gene from producing a product and can affect its phenotype. In 1998, Fire *et al* (1998) successfully elicited RNA interference in *Caenorhabditis*. Fast forward 25 years from Napoli *et al* (1990) the applications for gene silencing like PTGS, RNAi, & 'quelling' have been identified and observed in many eukaryotic organisms: plants, fungi, nematodes, insects and vertebrates (Whangbo & Hunter, 2008).

Much like PTGS, RNA interference (RNAi) is the sequence-specific gene silencing induced by double-stranded RNA (dsRNA) in animals (Zamore, 2001; Campbell & Choy, 2005). RNAi by dsRNA is accomplished through two primary pathways in the target organism: cell-autonomous & non-cell autonomous (Huvenne & Smagghe, 2009; Whangbo & Hunter, 2008). It must be also mentioned that examples of RNAi come from extracellular donations of dsRNA. This contribution of dsRNA from outside the target cells would be considered exogenous.

Accomplishing RNAi within a cell requires the presence of exogenous dsRNA. First, the dsRNA is introduced to the target cell, after which the dsRNA is cleaved by Dicer (RNase III enzyme) into small fragments of approximately 21 nucleotides (Hood, 2004; Whangbo & Hunter, 2008; Huvenne & Smagghe, 2009). These small nucleotide fragments of dsRNA are now known as short-interfering RNAs (siRNAs). The following mechanisms of cellular machinery are almost exclusively referencing the work by Novina and Sharp (2004). The RNA-induced silencing complex (RISC) separates the strands of the double-helix siRNA into sense and antisense strands. The sense strands are degraded and the antisense strands are used further in the process. At this point, siRNA can be involved in two pathways, depending on the host cell. Plant tissue & worms would require antisense siRNA strand to combine with RNA-dependent RNA polymerase (RdRP) gene product to start the endogenous proliferation of new dsRNA and a subsequent increase of siRNAs which would then encounter the RISC complex again to meld siRNA to messenger RNA (mRNA) and trigger mRNA degradation. Mammalian and insect cells do not possess RdRP siRNA proliferation machinery though an RdRP-like gene in *Drosophila melanogaster* (Price & Gatehouse, 2008) acts in a similar manner. This RdRP-like gene functions like RdRP and can produce new dsRNAs. The RISC complex re-enters the process and uses the antisense siRNA strand in the same manner: attachment of siRNA to mRNA for degradation and effective silencing of the gene silencing of the select mRNA transcript.

Exogenous dsRNA Options

Cell autonomous and non-cell autonomous are distinct components to RNAi. Cell autonomous RNAi is comprised of direct exposure of dsRNA to an individual cell to elicit silencing via the mechanisms described above. This method is site and cell specific. Depending

on the characteristics of the target cell, dsRNA may not be able to permeate the cell membrane and thus would require cell specific inoculation. Though the dsRNA in this case can be exogenous, it is not necessarily feasible for insect pest control applications. The focus of this section is to shed light on the non-cell autonomous uptake of dsRNA into cells to elicit a silencing effect.

There are two types of non-cell autonomous pathways of dsRNA introduction: injection & feeding (Whangbo & Hunter, 2008; Price & Gatehouse, 2008; Huvenne & Smagghe, 2009). The efficiency of these pathways is dependent on gene products of the *systemic RNA interference deficient-1/2* genes (*sid-1* / *sid-2*) (Price & Gatehouse, 2008; Huvenne & Smagghe, 2009). These genes allow for the passive transport of dsRNA into cells. The *sid-1* pathway is observed in non-gut cells of an organism. *Sid-1* is a multispan transmembrane that is absolutely imperative for systemic RNAi (Huvenne & Smagghe, 2009). The *sid-2* pathway was subsequently identified in the gut of *C. elegans* as a mutant to *sid-1* (Price & Gatehouse, 2008) and is responsible for dsRNA injected by oral uptake to accumulate in the gut cells via the lumen. Additionally, Whangbo & Hunter (2008) emphasize that *sid-2* proteins may have some synergistic effect on *sid-1* to assist in dsRNA transport in some models of its mode of action and site of action. These two pathways allow for the transport of dsRNA initially into the target cells and the systemic spread of dsRNA between like cells to induce a broad silencing effect.

Safety & Specificity

A benefit of RNAi-mediated efficacy against insect pests is the specificity in which insect and which cells within each insect are targets (Whyard *et al*, 2009; Price & Gatehouse, 2008). For instance, Price & Gatehouse (2008) report that a dsRNA created from a western corn rootworm (WCR: *Diabrotica virgifera virgifera*; Coleoptera, Family: Chyrsomelidae) cell line

had a significant impact on WCR larvae and two reported crop pests in the same Family Chrysomelidae: southern corn rootworm (SCR: *Diabrotica undecimpunctata howardii*) and Colorado potato beetle (CPB: *Leptinotarsa decemlineata*) due to sequence similarities of the targets cells in WCR, albeit the latter pests were effected at higher doses than WCR. Conversely, an additional pest cotton boll weevil (CPW: *Anthonomus grandis* Boheman; Coleoptera, Family: Curculionidae) was completely unaffected by the WCR dsRNA.

Though it is exciting to observe reproducible effects of RNAi in specific research, Scott *et al* (2013) regard RNAi technology as still a widely unknown process with potential risks to the ecosystem, insect resistance, target pest specificity, and non-target organisms. There is no denying that RNAi technology is in its infancy and that we have many more decades of research of various applications to gain clarity of what RNAi is truly capable of. At this time, examples are available to provide some defense to the concerns listed by Scott *et al* (2013).

Even though RNAi possesses a novel mode and site of action within an insect pest, it is worth considering the environmental impact if dsRNA for a specific pest was released and how long would the dsRNA persist in a field (if sprayed) or accidentally released in its true form (non-transgenic). Interestingly, the comparison between a recently published dsRNA for *Diabrotica virgifera virgifera* control, DvSnf7, possesses a half-life of approximately 30hrs depending on soil type (Dubelman *et al*, 2014) whereas a chemical insecticide, Malathion, for instance according to K. Newhart (2006) of the California EPA, recorded a half-life of 3 days in terrestrial applications. Even greater environmental persistence is recorded in the Material Safety Data Sheet (MSDS) of the FMC product Furadan ® 4 F (2001) has a half-life of 50 days on soil. The longer the half-life of a control measure in the environment can have deleterious effects on the environment and the immediate species within a community and lead to an

increase in selection pressure of a pest leading to resistance which is the beginning of the pesticide "treadmill"; a challenge to IPM that is actively avoided. The species specificity and potential short half-life of dsRNA (according to DvSnf7 references by Dubelman *et al*, 2014) offered by insect pest control through RNAi is worth the effort, though emphasis should be placed on not allowing history to repeat itself with insect pest control measures.

IV. Successful Gene silencing through RNAi of Agronomic Pests

The developing resistance to *Bacillus thuringiensis* (Bt) transgenic crops is a very real concern for many pest species (Gordon & Waterhouse, 2007; Tabashnik *et al*, 2013; Huang *et al*, 2014) which adds value to the species specificity gained by dsRNA expression by a host crop to mitigate insect pest damage. The benefits of RNAi are currently being investigated as a partial replacement of Bt in some cases. Environmental (exogenous, non-cell autonomous) RNAi transgenic products expressing species specific dsRNA are already being developed to control western corn rootworm (Baum *et al*, 2007). The Monsanto Company has published clear results on the reproducibility of environmental RNAi for the western corn rootworm (Baum *et al*, 2007; Bolognesi *et al*, 2012; Ramaseshadri *et al*, 2013; Dubelman *et al*, 2014). While the western corn rootworm was the primary target of the transgenic corn approach of dsRNA delivery, it did demonstrate efficacy against southern corn rootworm, SCR (Baum *et al*, 2007; Dubelman *et al*, 2014), which are also an occasional corn pest, though not as specified to a host as WCR (Purdue University, 2009). Incredibly, this was accomplished and published less than ten years after the first record of positive environmental RNAi results from Fire *et al* (1998) on the nematode *Caenorhabditis elegans*.

Western corn rootworm is not the only insect pest succumbing to RNAi. Researchers across the world are investigating many insect pests and attempting to elicit a reproducible gene

silencing in their target pests. As this digest gradually evolves into a discussion regarding the possibilities of implementing RNAi to control *S. frugiperda*, it must be stated that a very large, very comprehensive literature was published by Terenius *et al* (2011) that succinctly described the varying success of RNAi in Lepidoptera. It was a point by *this* author to avoid the aforementioned publication at all costs to avoid bias, though after some internal struggle the realization that this respectable review is four years old and is a lifetime ago given the speed of scientific progress. A selection of examples demonstrating Lepidoptera gene silencing through RNAi have been chosen to provide fuel for discussion.

Injecting dsRNA

Injection of dsRNA or feeding of dsRNA (non-cell autonomous-environmental RNAi) are the two most commonly encountered methods to induce a response of gene silencing a prospective pest species (Terenius *et al*, 2011). The benefits of injecting dsRNA into various insect life stages; egg, larvae, pupae, & adult are terrific to identify specific responses and gene silencing or simply to understand more about an insect's genome, like *Drosophila* (Bellés, 2010). Several Families of Lepidoptera have been examined for RNAi through *in vivo* (injection) exposure. Documented experiments confirming gene silencing in *Manduca sexta* (Family Sphingidae), *Bombyx mori* (Bombycidae), *Hyalophora cecropia* (Saturniidae), and several species of Noctuidae (i.e. *S. frugiperda, Spodoptera exigua, Helicoverpa armigera*) have been published.

Chen *et al* (2008) confirmed chitin synthase silencing through injecting dsRNA into late 4^{th} instar *S. exigua.* The result was a significant phenotypic reduction in overall volume ("stunting") of the treated larvae while the controls (uninjected & injected with buffer) were seemingly healthy. The cuticle of the treated insects was compromised, along with the chitin

supported trachea of the respiratory system. The compromised trachea would inhibit the development of tracheoles thus disrupting the effective "intracellular" respiration of various tissues (Klowden, 2013). Klowden's (2013) also states that trachea development is initiated by the protein products from the genes *branchless* & *breathless*, which would be a more acute target for RNAi. *Spodoptera exigua* was the target for RNAi by impairing development and immune responses (Surakasi *et al*, 2011). The focus of this experiment was to silence the production of integrin, a cell surface protein, which aids in microbe encapsulation during an immune challenge. When this specific integrin-β1- is silenced in 5th instar *S. exigua*, significant reduction in pupation rate and when pupation was successful, a pupal weight was observed.

The research conducted by Bettencourt *et al* (2002), investigated the innate immune system component *Hemolin* in *H. cecropia. Hemolin*, a gene product that binds to invasive microorganisms is also important to the development of embryos and correlates with ecdysone (molting hormone) peaks during larval and pupal metamorphosis. Bettencourt *et al* (2002) found that injecting *Hemolin* specific dsRNA into the pupa of *H. cecropia*, embryo development of the subsequent progeny were significantly deformed. The conclusion being that dsRNA not only can effect a current generation of insects, but also the next generation, which could be a popular consideration for reproductive biology and control in the field.

Wang *et al* (2013) investigated the response of *H. armigera* adults after pupal injection of dsRNA specified for HMG-CoA (HMGR) silencing. The reduction in viable offspring is a very interesting perspective and option in the quest for pest insect control. HMGR is one such gene product that has its "fingers" in many aspects of insect physiology. Wang *et al* (2013) provide examples of HMGR facilitating pheromone production, embryonic development and vitellogenesis. Vitellogenesis is the production of vitellogenin (yolk protein) from the fat body

and transferring these proteins into the developing oocyte of female insects (Klowden, 2013). The silencing of either three of these physiological products could impede fecundity or mating behavior which would present an ultimate decrease of offspring within the environment. In the experiments by Wang *et al* (2013), HMGR and vitellogenin gene product expression were significantly reduced by the silencing of HMGR. The fecundity was reduced by a staggering 99% (compared to the negative controls) under the expressed results!

The last example to share was conducted by Griebler *et al* (2008) on injecting *S. frugiperda* adults & larvae with dsRNA capable of silencing the production of allatoregulating neuropeptides. Allatostatin and allatotropin are produced by the brain of insects to discourage or stimulate corpus allatum (CA) production of juvenile hormone, respectively (Klowden, 2013). Juvenile hormone (JH) is a highly versatile hormone that contributes to immature growth regulation, reproduction, metabolism, and diapause (Klowden, 2013). Griebler *et al* (2008) demonstrated silencing allatostatin (AS) and allatotropin (AT) production in adult female *S. frugiperda* would be advantageous to reduce fecundity by approximately 40%. Injections of dsRNA to silence AS & AT were performed on 5th instar *S. frugiperda*. Both cases of AS & AT silencing led to an increase in larval time to pupation when compared with the controls. Silencing of allatostatin, or the silencing of the silencing of JH, lead to an increase in peak larval weight that was not observed in the control which meant that the larvae were still feeding vigorously. Allatotropin silencing (which is basically the presence of allatostatin) retarded the growth and weight of dsRNA treated larvae when compared to the control, in some cases it was significant.

Oral Delivery of dsRNA

Gene silencing through feeding has potential utility in IPM (Terenius *et al*, 2011; Ivashuta *et al*, 2015). Select research demonstrates that it is indeed possible to silence gene expression through feeding dsRNA to certain pests. Surakasi *et al* (2011) also examined feeding with the previously discussed dsRNA injection. In the feeding component of the experiments, Surakasi *et al* (2011) found that orally exposing *S. exigua* to dsRNA to inhibit β1-integrin production in the midgut epithelium of growing larvae caused significant mortality in a dose-response based assay of dsRNA coated cabbage leaf. Griebler *et al* (2008) also performed a feeding bioassay on *S. frugiperda* separately from their injection assay to inhibit allatoregulating neuropeptide expression. The oral uptake of dsRNA resulted in a decreased expression of allatostatin (22.3 ±9.6%) and allatotropin (3.3±2.3%), even though no corresponding phenotypic responses accompanied the CA products' knockdown. The interpretation of the research by Griebler *et al* (2008) is more of a boost to possibilities than a process available for IPM implementation. A JH mimic or regulator by dsRNA would be more impressive demonstrating its effect on <2nd instar *S. frugiperda* larvae. Dow (1992) produced results regarding a decline in alkalinity of lepidoptera midgut pH before the 4th instar. This decline in pH could benefit dsRNA uptake by the apical cells of the midgut before degradation occurs.

A prime target for RNAi by feeding is the insect midgut (Huvenne & Smagghe, 2009). The midgut uptake of dsRNA is much like that of Bt (Hernandez-Rodriguez *et al*, 2013). There is a benefit of any uptake of dsRNA by the apical midgut cells. The midgut of many holometabolous insects is the only portion of the alimentary canal that does not possess a chitin-based cuticle to protect the animal from injury during ingestion (pharynx & foregut) or expulsion (hindgut) of its' selected host tissue. Uptake would either silence target cells of the midgut; or transport through the midgut to the hemolymph and locate the target cells. Oral delivery of

dsRNA to *Epiphyas postvittana* (Lepidoptera, Family: Torticidae) by Turner *et al* (2006) suppressed the pheromone binding protein gene (PBP1) and carboxylesterase gene (CXE1) found in the antennae & midgut, respectively. Turner et al (2006) demonstrate statistically significant differences between the controls and RNAi response of the insect larvae during the first 7 days of exposure after an oral dose.

Toprak *et al* (2013) investigated the effects of multiple genes contributing to the formation of the peritrophic matrix (PM) by feeding dsRNA to *Mamestra configurata* (Lepidoptera, Family: Noctuidae) larvae. Toprak *et al* (2013) used both 4th instar larvae and neonate (newly eclosed from egg) *M. configurata* in their experiments. Depending on the gene of interest, silencing was confirmed within 24hrs or up to 72hrs post-feeding or silencing was not significantly achieved. Neonate *M. configurata* were highly susceptible to CDA1 dsRNA and no CDA1 expression was observed in the first 24-48hr depending on dosage. However, CDA dsRNA exposure to 4th instars some silencing but not to the extent as neonates. Unfortunately, no phenotypic responses were described other than CDA dsRNA against neonates *M. configurata* and the impacted behavior at high doses. The most enlightening portions of this experiment were the impact to the PM forming genes and that a research used neonate larvae as a more appropriate life stage to gauge efficacy than later instars, as seen in the affect was greater in neonates than 4th instars of *M. configurata* in these experiments.

Keeping with the trend of midgut target cells, Bautista *et al* (2008) explore the possibilities of cytochrome P450 suppression in the diamondback moth, *Plutella xylostella* (Lepidoptera, Family: Plutellidae) to mitigate aspects of resistance. The focus of this experiment was to observe the response of a permethrin (a synthetic insecticide) resistant *P. xylostella* strain to dsRNA specifically to silence the genes responsible for overexpression of cytochrome P450

by the *CYP6BG1* gene. Cytochrome P450 is family of enzymes produced by the fat body and midgut (Snyder *et al*, 1995) that play important roles in insect metabolism and gut defense of plant secondary metabolites (allelochemicals) and insecticides (Scott, 1999; Li *et al*, 2002). Larvae fed *CYP6BG1* dsRNA were exposed to permethrin. The once permethrin-resistant *P.xylostella* larvae were significantly impacted by permethrin once *CYP6BG1* suppression occurred. Cytochrome P450 has been identified in several economically important species and the work cited by Bautista *et al* (2008) offers an interesting perspective to mitigate insecticide resistance among Lepidoptera agricultural pests.

In 2013, Asokan *et al* (2014) analyzed the response of cotton bollworm, *Helicoverpa armigera* against five dsRNAs: glutathione-S-transferase (GST- a type of detoxification enzyme (Yu, 1989)), cytochrome P450, trypsin & chymotrypsin (both serine proteolytic enzymes found in ~95% of Lepidoptera (Asokan *et al*, 2013)), & juvenile hormone acid methyl transferase-*jhamt*-a metamorphosis requirement. Neonate *H. armigera* were fed dsRNA preparation for each of the described gene products and silencing confirmed in both gene suppression and phenotypic responses by the treated larvae. Larvae exposed to GST, P450, trypsin, chymotrypsin, and *jhamt* elicited significant weight reductions compared to the controls. Pupal weight was slightly affected by only some of the target genes (*jhamt &* P450). This profound experiment by Asokan *et al* (2014) was one of the most exciting examples of RNAi in Lepidoptera, specifically in a polyphagous Noctuid species this author encountered during the literature search and review. It provides several opportunities for investigation for successful and reproducible environmental RNAi in *S. frugiperda*.

V. The Fall Armyworm & Resistance to IPM Tactics

Biology & Behavior

Spodoptera frugiperda is a highly destructive polyphagous noctuid pest that resides in a range from Argentina S.A. to the United States of America (Santos *et al*, 2003; Murúa & Virla, 2004; Nagoshi & Meagher, 2008; Schöfl *et al*, 2009; Hernández-Rodríguez *et al*, 2013) and even Canada (Huang *et al*, 2014). According to Capinera (2005), *S. frugiperda* undergo complete metamorphosis (holometabolous) with four distinct life stages: egg, larval, pupa, & adult, two of which are free living-larva and adult. The larval stage of *S. frugiperda* is the economically damaging life stage. Within the larval life stage, 6 instars (growth stadia) allow the larvae to exponentially increase its herbivory with every subsequent instar until its 6^{th} and last where it exhibits pre-pupation behavior (wandering; searching for adequate pupation location). The larvae of *S. frugiperda* are a significant pest of corn, sorghum, cotton, turf grass, and rice varieties. In the evolutionary arms race between plant defensive allelochemicals to discourage insect feeding and insect midgut produced enzymes to combat these plant secondary metabolites (Zhu-Salzman *et al*, 2008). If scenario holds true, *S. frugiperda* seems to be winning.

Nagoshi & Meagher (2008) acknowledge & reference two separate strains of *S. frugiperda* with the preferences for corn & rice, respectively. These two morphologically similar strains that have different host plant requirements but can still mate with each other will continue to propagate this behavior: Corn-strain & rice-strain (Schöfl *et al*, 2009). As a multivoltine pest, *S. frugiperda* produce multiple generations per year when the climate is favorable, such as the tropics & subtropics of South America, the Caribbean, and the southeastern United States. Yearly migrations of the resident *S. frugiperda* of southern United States reach into parts of the United States east of the Rocky Mountains that cannot sustain multivoltine behavior, as *S. frugiperda* lacks the ability to diapause (Capinera, 2005; Nagoshi & Meagher, 2008).

Resistance to IPM Tactics

Young & McMillian (1979) first reported on the resistance of *S. frugiperda* to carbaryl, a chemical insecticide. Over the next three decades resistance has been identified in *Spodoptera frugiperda* both in the laboratory and the field to various IPM tactics on which we rely. One mechanism for resistance occurs through an increase in 'selection pressure' (Pedigo & Rice, 2009) where some control method may kill 99% of all of its target pests, but that 1% may possess some innate ability to withstand that selection pressure. The progeny from that 1% of survivors will pass along those innate abilities to withstand the selection pressure, and so on, creating an opportunity to build a resistant population. In the case of *S. frugiperda,* multiple generations per year suddenly take those 1% of survivors to found a new population of resistant insects that are completely uninhibited by traditional control measures. Mistakes such as ineffectively controlling 100% of the population will lead into crop loss or environmental damage to increased use of the selection pressure, or both. A few select examples of *S. frugiperda* resistance to chemical, biological, and cultural (transgenic) insecticidal methods are provided below.

Chemical

In 2013, Carvalho *et al* reported on the comparison of potential gene products for resistance between two suspected resistant strains and a control of *S. frugiperda*. In this experiment, many enzyme products suspected or confirmed to offer some component to insecticide resistance were tested for expression levels. The over-expression of certain genes that produce the complex of enzymes was observed in the 'resistant' strains of *S. frugiperda*, whereas low expression was frequently linked with the 'susceptible' *S. frugiperda* strain. Carvalho *et al* (2013) reference the examined enzyme products are the mechanisms that underlie resistance to insecticides like pyrethroids, carbamates, & organophosphates.

Biological

Natural enemies like predatory insects, vertebrates (birds & mammals), and microorganisms can achieve some control on *S. frugiperda* populations (Capinera, 2005). However, endemic natural enemies wouldn't be able to follow adult *S. frugiperda* during migrations. Fungal, bacterial, and viral pathogens can provide some *S. frugiperda* control, though it is varies by community and life stage (Capinera, 2005). Interestingly, Fuxa & Richter (1988) were able to develop a *S. frugiperda* colony that was resistant to the effects of the Fall Armyworm-specified Nuclear Polyhedrosis Virus (NPV). In as little as 7 generations the authors achieved a resistance ratio of NPV inclusion bodies three times higher than what would affect a susceptible *S. frugiperda* strain. The NPV resistant *S. frugiperda* colony did, however, exhibit reduced fecundity when compared to a susceptible strain after >20 generations, though the time to complete development was very similar.

Transgenics

Transgenic crops are engineered to express a gene of interest from one organism and combine it with the genome of a different host organism so that the host organism can express this 'new' gene during its life processes to enhance a selected attribute (Colorado State University, 2004). In the case of Bt transgenic corn, select insecticidal *Cry* (crystal) protein producing genes from the bacteria *Bacillus thuringiensis* are selected and artificially incorporated into a corn plant to express the insecticidal characteristics of the Bt *Cry* proteins. Depending on the view point, transgenic crops could be considered to be both biological and cultural control methods: biological due to the additive features of implanting a gene from a ubiquitous bacteria like Bt and its various strains that can achieve insecticidal results in their wild-type form and cultural because of the relatively recent use of transgenic crops as means to

facilitate IPM as a "smart bomb" to alleviate catastrophic insect damage year to year. No matter how elaborate the science, it seems, life-or insects- will find a way around our control tactics. Storer *et al* (2010) presented evidence to this fact by the inheritance of Cry1F resistance of *S. frugiperda* populations in Puerto Rico was up to 450 fold more resistant than laboratory strains of *S. frugiperda* when exposed to Cry1F.

In 2014, Huang *et al* reported one of the first instances of Bt resistance of *S. frugiperda* in mainland United States (Florida). Resistant *S. frugiperda* were collected from non-Bt corn in Florida that possessed an extremely high allele frequency for Cry1F, a Bt toxin that is highly efficacious to S. *frugiperda*. The Florida *S. frugiperda* sample with higher than expected resistant allele frequency was almost three times greater than a separate population of S. frugiperda from Louisiana. This in itself is noteworthy from a population genetics standpoint of genetic drift: the greater the allele frequency in a population, the greater the probability of the subsequent generations retaining that resistant allele to the point of fixation, which would result in an entire population possessing the resistant allele (Hamilton, 2009). The fixation event would likely take many, many generations, but the probability of the Cry1F resistant allele presence within a population would increase. Upon further investigation of potential field resistance the Southeast United States, several selected populations of *S. frugiperda* larvae were collected from both Bt and non-Bt corn and tested against Cry1F toxin as F2 neonates. The resulting resistance ratios exhibited by these collected strains of *S. frugiperda* were 3 to >270 fold higher than the susceptible strain. This is an alarming level of resistance considering the first registered Cry1F products were released in the United States in 2001 (Huang *et al*, 2014). Huang *et al* believe that one contributing factor to Cry1F resistance in the U.S. is due to the migratory behavior of *S. frugiperda* from Puerto Rico. The island territory of Puerto Rico is

likely subject to allopatric speciation, a component of evolution, where gene flow is limited to a set of organisms in the isolated area (like an island) due to lack of new genetics. Any resistance that originates in Puerto Rico to Cry1F will quickly lead to the dominance of a resistance allele without adequate genetic exchange of susceptible *S. frugiperda*.

VI. Is *S. frugiperda* RNAi Plausible?

Critique of Cited Research Examples

There numerous hurdles to elicit the same response in *S. frugiperda* (and all Lepidoptera) when compared to the success for some Coleoptera pests like *D. v. virgifera* (Baum & Roberts, 2014). Baum & Roberts (2014) discuss these barriers in depth and reference the *S. frugiperda* as recalcitrant or uncooperative. It is no surprise that the midgut of *S. frugiperda* is a hostile environment for dsRNAs, but Baum & Roberts (2014) also state that the hemolymph of *S. frugiperda* also rapidly degrades dsRNAs as a response to Lepidoptera viruses. This seems likely due to the responses (Fuxa & Richter, 1988) experienced in 'engineering' an NPV resistance *S. frugiperda* colony. It becomes unavoidably apparent the research summaries provided by Terenius *et al* (2011) and Baum & Roberts (2014) presents significant difficulty for this author to present some novel concept of *S. frugiperda* control through RNA interference, but then again, this author is not known for 'throwing in the towel'.

Research on *Helicoverpa armigera*, another polyphagous noctuid pest, has experienced incredible success in RNAi-mediated control when compared to *S. frugiperda*: 11 cited success of mRNA silencing & 10 of those instances resulted in phenotypic impact (mortality or stunting) on *H. armigera* versus 3 cited success of only mRNA silencing with no reported phenotypic impact (no mortality/stunting) on *S. frugiperda* (Baum & Roberts, 2014). Baum & Roberts (2014) also produced information of *Spodoptera exigua* RNAi success that resulted in 3 cited

experiments that observed phenotypic impact, 2 of which elicited mRNA silencing. These presentations of success provide an immediate point of emphasis for *S. frugiperda* experimentation.

All three of these pest examples are polyphagous noctuids, and even a shared genus (*Spodoptera*) is present. There must be some similarities in physiology between *S. frugiperda* & the likes of *H. armigera* and *S. exigua*. Three significant factors have become apparent.

First, the cited *S. frugiperda* successes in mRNA silencing were on valid targets, but at fourth and fifth instar, these insects have an increasingly strong immune system after 3rd instar (Klowden, 2013). All *H. armigera* experiments occurred on neonates, second instar, or third instar larvae. Two of *S. exigua* experiments were conducted on <3rd instars. The third success on *S. exigua* was by Surakasi *et al* (2011) that targeted the β1-subunit integrin which provides a component to cellular immunity. This target life stage for β1-subunit integrin silencing by Surakasi *et al* (2011) was 4th instar, the subsequent instar where Klowden (2013) suggests that the innate immune system develops rapidly after a spike in 20-hydroxyecdysone (20E) to facilitate the proliferation, differentiation, and dispersal of hemocytes from lymph glands. While Baum & Roberts (2014) and this author (though in a limited capacity) describe these cited successes between *S. frugiperda, S. exigua*, & *H. armigera*, a correlation this author observed seems to be missed (intentionally or unintentionally). The *S. frugiperda*, like many other Lepidoptera larvae, experience significant increases in body volume, head capsule width, and feeding capacity (personal observation; Capinera, 2005) from 3rd instar to 6th instar. The accelerated larval growth and 20E spike that triggers immune system robustness conceptually correlate. Logically, an insect with a higher feeding capacity may have a higher probability to encounter pathogens or plant toxins that could debilitate the larvae. An increase in size could be

reliant on the immune system. This correlation brings clarity as to why the work of Surakasi *et al* (2011) on 4th instar *S. exigua* by β1-subunit integrin silencing was the only listed 4th instar success of Lepidoptera by Baum & Roberts (2014): The immune system & larval instar is imperative to RNAi exposure for lepidoptera.

Second, the gene silencing targets and exposure tactic may not have been optimal for S. frugiperda phenotypic responses. Midgut detoxifying enzymes, immune system components, ecdysone receptors, molting regulators, acetylcholinesterase (AchE) receptors, and neurohormone regulation are all targeted across these three pests: *H. armigera* & *S. exigua* than *S. frugiperda* (Baum & Roberts, 2014). The results posted by Baum & Roberts (2014) for *S. frugiperda* RNAi success are isolated to a serine protease and allatoregulating peptides for the synthesis of juvenile hormone. Messenger RNA was reported to be suppressed in *S. frugiperda*, however no significant phenotypic response was glaringly apparent to be considered [Griebler *et al* (2008) reported reduced larval weight, but no instar differentiation to the control through droplet feeding] by Baum & Roberts (2014). This could be again due to the presence of a stronger immune system and late instar exposure. The serine protease inhibitor did not elicit recorded stunting or mortality by Baum & Roberts (2014) in *S. frugiperda*, however other detoxifying enzymes like cytochrome P450 inducing genes (*CYP6AE14 & CYP6B6* genes) were observed to have a significant phenotypic response on *H. armigera* when exposed to both transgenic plants and artificial diet at 3rd instar. It is this author's speculation that droplet feeding may not be an appropriate method of oral delivery of dsRNA to *S. frugiperda*. Transgenic plants, leaf tissue, and artificial diet were the means for dsRNA delivery to *S. exigua* and *H. armigera*. This author suspects that with the delivery of a dsRNA droplet to *S. frugiperda* is putting complete focus of the immune system and any detoxifying enzymes present on the

dsRNA. Leaf tissue or artificial diet may provide some 'cloaking' attribute to the dsRNA when digestion is initiated.

Third, cloaking or protecting double-stranded or short-interfering RNAs for delivery to *S. frugiperda* may be an option (as well as other recalcitrant pests) for successful dsRNA uptake (Baum & Roberts, 2014). Some of these agents include siRNA variants (which is the actual mRNA silencing factor) and nanoparticles to protect dsRNA (Baum & Roberts, 2014). One aspect not specified by Baum & Roberts (2014) that this author found some value was the cloaking of *Metarhizium anisopliae* (a ubiquitous entomopathogenic fungi) hyphal bodies by a collagenous protective coat that is not recognized by the immune system of *Manduca sexta* (tobacco hornworm, Lepidoptera Family: Sphingidae) (Wang & St. Leger, 2006). The protective coat, deemed MCL1 (*Metarhizium* collagen-like protein), codes for an antiadhesive protein that is seemingly impervious to hemocyte encapsulation (Wang & St. Leger, 2006). Wang & St. Leger (2006) did not provide any indication of an oral delivery system; rather, all insects exposed in this research were done so by injection of MCL1 hyphal bodies into the hemolymph. This information, while interesting, is solely dependent on a coat protein protecting dsRNA (or siRNA) sufficiently in the midgut after ingestion of the target pest so ds/siRNA uptake is possible by the gut apical cells.

Though the examples of *H. armigera* & *S. exigua* absolutely do not encompass the actual mode of action or site of action in *S. frugiperda*, but differences are mounting in the execution of *S. frugiperda* RNAi attempts. This author did not find any research literature attempting to duplicate the RNAi successes for *H. armigera* & *S. exigua* on *S. frugiperda*, even though the newest document on *S. frugiperda* RNAi success was from 2010 (Baum & Roberts, 2014). The use of protective packaging of ds/siRNAs on *S. frugiperda* was also not observed during the

literature search for the summary though opportunities in this developing technology are exciting. This author recommends that these examples of RNAi success on non-*S. frugiperda* noctuids and delivery agents should be absolutely investigated further.

Additionally, a barrier regarding the strain of *S. frugiperda* used during experimentation may cause some variability that could confound bioassays. How an *S. frugiperda* colony is reared under controlled conditions may contribute to knowledge of the pest and what nuances the colony possesses. Not all artificial medias are standard or possessing all necessary dietary requirements for uniform development from neonate to adult. Some artificial medias this author has worked with impact the successful number of instars at a certain temperature or reduce the number of successful pupae in the controls. This information itself can confound bioassays. It would be a very important component to any experiment to have a compilation of data regarding instars & attrition at each time point (day) in a control to gauge the success of an experiment. If a colony of *S. frugiperda* is maintained properly, this information could shed light on any anomalies during the experiment for validation to a phenotypic impact.

Lastly, the information provided in many of the research articles that were reviewed by this author did not specify some of the finer details of an experiment. This elevates concerns about the reproducibility in unconnected research duplications and success by 'sheer luck'. It is in the opinion of this author that any researcher from academics to industry or from undergraduate to post-doctoral should be able to follow the experimental outline to elicit a similar, if not identical, response in research. Only then can the scientific community truly validate a mode and site of action of *S. frugiperda* RNAi, among other recalcitrant Lepidoptera pests.

Worthy Gene Targets of Investigation

Baum & Roberts (2014) discuss that *D. v. virgifera* is relatively alone when it comes to sensitivity to certain dsRNAs that target cells 'housekeeping' genes and that target gene selection is not necessarily the key to breaking through any target pests' physiological barriers. This author believes this statement. A lack of supporting literature regarding successful phenotypic response; i.e. stunting or mortality, in the case of *S. frugiperda* it could be that we haven't experienced significant RNAi induced response/event yet. It would be in the interest of a research proposal to screen dsRNAs against *S. frugiperda* through a dietary complex to observe the effects on the life cycle.

The insect fat body is quite possibly one of the most important tissues to investigate. The fat body contributes immune system responses, vitellogenin production, metabolism, detoxifying agents like cytochrome P450 and growth coordination, and energy storage (Snyder *et al*, 1995; Klowden, 2013). Interestingly, Ramaseshadri *et al* (2013) specifically cite the effect on *D.v. virgifera* fat body from the DvSnf7 dsRNA along with the midgut for significant impact on their insect pest. Investigating the constituents of the fat body would be worth the effort, especially during life cycle studies due to the slow acting nature of RNAi (Baum & Roberts, 2014).

The research cited makes it difficult for this author to state that highly efficacious RNAi in *S. frugiperda* is currently attainable, perhaps impossible, but the same research has provided some opportunities for investigation. Among these opportunities are target genes that seem to play an integral role in insect life processes if they were to experience RNAi silencing. The damaging stage of *S. frugiperda* is an immediate focus, though there would be excellent potential for reproductive impact. Klowden (2013) describes such targets and a select few materialized to this author as worthy of investigation.

1. *Dumpy*: an extremely large extracellular matrix protein that is expressed at multiple sites for muscle and tracheal attachment. Result of silencing: muscle and tracheal function would be negatively impacted.
2. *Branchless & Breathless*: genes that are imperative to the formation and function of the trachea & tracheoles. Result of silencing: Suffocation.
3. *Resilin*: an important elastic protein that contributes to locomotion, alimentary contractions and capabilities for cross-linkage with other proteins (like *Dumpy*). Result of silencing: Locomotion and structure of the larvae and/or adult could be negatively impacted.
4. *Lipophorins*: transport proteins responsible for shuttling lipids from the fat body to other cells for cell synthesis and some transportation of JH. Result of silencing: Cellular recruitment of lipids, energy, and hormone communication would be disrupted.
5. *Insulin-like Growth Factor (IGF)*: IGFs acts on the prothoracic gland to increase PG growth and eventually PTTH/ecdysone cascade. Result of silencing: Halting this process would cease molting behavior.

Potential Opportunity of RNAi in IPM Control Tactics

It may take time to truly be certain of the possibility of an efficacious mode and site of action are discovered for *S. frugiperda.* The increasing evidence for resistance against many of Mans' efforts to control this pest has been thwarted and We, as researchers and scientists in the realm of agriculture, are in what could be the 11th Hour. Though it is an assumption based on faith of the collective intelligence possessed by the IPM community; We must be ready to

implement control tactics once we have the ability to silence target genes of *S. frugiperda* through RNAi.

The mechanisms of interested have been described not only in this review but by Terenius *et al* (2011) and Baum & Roberts (2014). There are tactics that could be relevant to battle *S. frugiperda* in the field. Several IPM concepts could be utilized when to an approved and successful RNAi mechanism for *S. frugiperda*. Two tactics seem the most feasible for RNAi given the current technology: transgenic crops & a sprayable biopesticide.

Baum *et al* (2007) successfully elicited dsRNA production in corn to achieve gene silencing in *D.v. virgifera*. Further work by Baum & Roberts observe that when 'stacking' an efficacious *Cry* toxin and dsRNA into a plant genome a synergism is observed and impact on the insect is greater than the additive effects of the separate components. It would be advantageous to consider these 'stacked' transgenic products with a variety of "ammunition", providing at least 2 novel modes of cellular action to mitigate resistance, which, as it has been discussed, is a very real problem regarding *S. frugiperda* in the field to both transgenics and insecticides. This, of course, is only if enough *Cry* toxin & ds/siRNA can be produced by the host plant to be highly efficacious. Any reduction of expression of the insecticidal products will provide an opportunity for a resistance selection pressure.

Baum & Roberts (2014) also allude to a topical application for RNAi-based pest management strategy. Though previously described, the key to this concept would be the protection of ds/siRNAs from the environment until the target pest (*S. frugiperda*) can imbibe the treated plant tissue. Nanoparticles of siRNAs would be a likely vector for any topical application. There are concerns over environmental stability of this RNAi tactic range from

ultraviolet light, plant exudates & microbial degradation which is similar to what plagues chemical insecticide in the field (Baum & Roberts, 2014). A couple of additional concerns mount when considering an RNAi biopesticide: effort & siRNA nanoparticle production. A benefit of transgenic crops is that a grower is paying a company for both the seed & insecticidal capabilities and reduces the reliance and time on buying and applying chemical insecticides except in extreme cases. An RNAi biopesticide may not last long in the environment, so extra effort in scouting fields and calculating economic thresholds and injury levels (ET & EIL) may be a considered a burden to particular growers and the result may be spraying an RNAi product at pessimal time for pest's larval/damaging life stage interaction or indiscriminant use of the spray. The concentration at which the RNAi spray will be effective bears the burden of both cost and field efficacy. If a 1000ppm concentration is required to get an optimal effect to *S. frugiperda* any deviation from this will ignore IPM methodologies (Pedigo & Rice, 2009) but to achieve the 1000ppm concentration to be commercially available may be far more expensive (Baum & Roberts, 2014) than a synthetic insecticide counterpart.

Pheromone bait traps can be mentioned in the same breath as a sprayable RNAi product for *S. frugiperda* control. Pheromones are chemical signals that allow members of the same species to communicate in a variety of ways: reproduction/mating, alarm, location, aggregation & repellents (Pedigo & Rice, 2009). If a novel mode of RNAi action on *S. frugiperda* were identified it would be an opportunity to use aggregation pheromones to attract females to an RNAi bait with a ds*Hemolin* (Bettencourt *et al*, 2002) plus ds*HMGR* (Wang *et al*,2013*)* and hopefully elicit some reduction in either egg production or embryo development. If an adult male *S. frugiperda* specific dsRNA is identified for spermatogenesis, it could be useful in a bait format, also using female *S. frugiperda* sex pheromones. Pheromone baits that are highly

volatile could not only alter male behavior but also to sterilize the male and impede female fecundity in that manner. Baits, however, would require oral uptake of the RNAi agent. Lifespan of adult moths can be limited to only a few days with *S. frugiperda* even under optimal conditions (personal observation, unpublished results).

Sterile insect technique may be an acceptable avenue for RNAi if a male-specific gene can be effectively suppressed in *S. frugiperda*. Sterile insect technique is documented in a number of species: fruit flies, moths, mosquitos, tsetse flies and screwworm flies (Food and Agriculture Organization of the United Nations, 2014). The FAO describes that male insects are subjected to radiation to sterilize them; the sterile male insects are then released into a population to mate with females. The same outcome could be accomplished via RNAi sterility by exposing late instar larvae to a dsRNA diet, silencing the downstream spermatogenesis during pupation and release the pupae into the environment. Benefits would include an inundation of non-damaging life stages that have a flight capability for searching behavior and can perhaps outcompete feral, reproductive male *S. frugiperda*. Hindrances of this tactic would be cost of dsRNA and insect larval production, which would require significant infrastructure to encompass the range of *S. frugiperda* between 2 continents, Central America, and the Caribbean.

VII. Concluding Statements

Hindsight is almost always 20/20. The face of insect control history is marred with scars, each one a lesson. Errors in insect control of the past must be evaluated and corrected to the best of our ability to make gains in the future. One of the foundational remarks of Stern *et al* (1959) was to learn about the pest. While more in a context of sampling and understanding the community organization of trophic levels, its lesson is in knowledge. It is our responsibility as scientists to ethically scrutinize barriers with vigor and collaborate willingly and without

restriction to approach problems systematically. This cannot be emphasized enough. In many cases we are doing this. Strong research and reviews of a research topic are readily available. Because of this, the knowledge is accumulating over the phenomenon of RNAi. In the literature cited for this review, many different techniques have been attempted, some with mixed results, but the effort in the research is there and it is gaining momentum.

There are potential opportunities for *S. frugiperda* RNAi, though; but the results must be reproducible. Successes of RNAi by a variety of methods against other polyphagous Lepidoptera species may be leveraged to assist in *S. frugiperda* control tactics. The key is in reproducible uptake of exogenous dsRNA. The odds are stacked against us for an efficacious delivery system for *S. frugiperda* and other difficult insect species at the moment, at least at an economically or metabolically feasible rate. The technology concerning RNAi is still in its infancy when compared to Bt. *Bacillus thuringiensis* has been acknowledged as an insect control tool for over a century (Bravo *et al*, 2013) and any efforts to run before we can crawl regarding RNAi will result in catastrophe. It may be 5-10 years longer before another significant breakthrough occurs in research on the insecticidal applications of RNAi, at least one that includes *S. frugiperda*.

All of the proposed research and tactics for RNAi efficacy are examples to pressure researchers around the globe to continue focus on Lepidoptera RNAi, but more importantly, to refocus and delve into *S. frugiperda* RNAi and verify its use as a plausible component to IPM practices. It is of the utmost importance that an efficacious novel mode of action be identified and produced to mitigate any further *S. frugiperda* resistance to our current chemical, biological and transgenic control methods for this pest.

"We keep moving forward, opening new doors, and doing new things, because we're curious and curiosity keeps leading us down new paths"-Walt Disney

VIII. Epilogue: Author's Statement

This degree project was meant to challenge me significantly. Through my last 8 years in corn insect pest control with DuPont-Pioneer and my coursework for this degree I have tried to focus on IPM. Integrated Pest Management fascinates me because of its complexity and multiple interactions. Integrated pest management was to serve as backbone for methodologies and thought processes regarding the importance of RNAi & its plausible effect on *S. frugiperda*. I made mention of it several times, but based on the literature, *S. frugiperda* RNAi is impossible right now. So, in 9 weeks, I did my best to learn, digest, and regurgitate the available information that has been the life's work for some of the cited scientists to provide a quality discussion over the options that may still be available to experiment, which in itself, is a long shot. I readily utilized information from several of the courses I have taken, which was hopefully visible in my references and writing. This topic, *S. frugiperda* RNAi is something that I would be eager to work with given the opportunity due to its challenges and that it would force me to reduce my knowledge and experience deficiencies with molecular biology and biochemistry. Thank you to all of my instructors to introduce incredibly helpful and fascinating material to help me retain a healthy knowledge of entomological concepts and leaves me craving more.

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